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13. ABSTRACT ***(Maximum 200 Words)***

The human *CHES1* (checkpoint suppressor 1) gene is sufficient to restore DNA damage-induced G_2 arrest in multiple *S. cerevisiae* checkpoint mutants by activation of an alternative checkpoint pathway. Our goal is to identify the *CHB* (checkpoint bypass pathway) genes that constitute this alternative checkpoint, to isolate the human counterparts of these genes, and to compare their structure and activity in normal and cancer tissues.

In an effort to identify the genes involved in the alternative pathway, we performed a comprehensive screen in *S. cerevisiae* by mutagenizing a *cdc9-8*, *rad9Δ* mutant strain, which is both UV-resistant and grows at 30°C in the presence of *CHES1*. The screen resulted in three mutants that display varying degrees of temperature- and UV-sensitivity. In summary, *chb13* is a strong mutant in which *CHES1* can no longer suppress either the temperature- or UV-sensitive phenotypes. *chb16* and *chb57* are highly temperature-sensitive/moderately UV-sensitive and highly UV-sensitive/moderately temperature-sensitive, respectively. Despite multiple attempts, we were unable to identify the gene(s) mutated in the *chb* mutant strains.

We have taken an alternative approach to this goal by using an *S. cerevisiae* insertional mutagenesis method, which will avoid the cloning problems experienced in using the previous EMS methodology. In addition, in efforts to identify CHES1-interacting proteins by biochemical methods, we have constructed a human *SOS1* (son of sevenless)-*CHES1* bait construct and a GST (Glutathione-S-Transferase)-*CHES1* fusion construct for use in a cytoplasmic yeast-two-hybrid screen and GST pulldown experiments, respectively. The insertional mutagenesis screen, the two-hybrid screen, and the pulldown experiments are currently in progress.

14. SUBJECT TERMS
CHES1: checkpoint suppressor 1; EMS: ethylmethane sulfonate
CHB: checkpoint bypass pathway; 5-FOA: 5-fluoroorotic acid

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Introduction

Eukaryotes possess well-conserved responses to DNA damage, including DNA damage-induced cell cycle arrests or "checkpoints" (reviewed in Hartwell and Weinert, 1989; Zhou and Elledge, 2000). The genes responsible for checkpoint function control transitions between the different cell cycle phases. When DNA damage occurs or replication is blocked, checkpoints arrest the cell cycle allowing replication and repair to take place. In order to identify genes required for the human G_2 checkpoint, our laboratory performed a high-copy suppressor screen of a human library to identify human cDNAs that suppress *S. cerevisiae* mutant phenotypes resulting from mutations in either *RAD9* or *MEC1* (Pati *et al*, 1997). One cDNA, *CHES1* (checkpoint suppressor 1), was identified and shown to suppress checkpoint phenotypes observed in multiple checkpoint-deficient strains.

In our model, wild-type *S. cerevisiae* strains are resistant to DNA damage because of the presence of both the primary *MEC1*-dependent checkpoint pathway and a less active, alternative *MEC1*-independent pathway. Disrupting the *MEC1*-dependent pathway results in strains that exhibit checkpoint phenotypes, and expression of the CHES1 protein suppresses those phenotypes by activating the alternative pathway in yeast (figure 1).

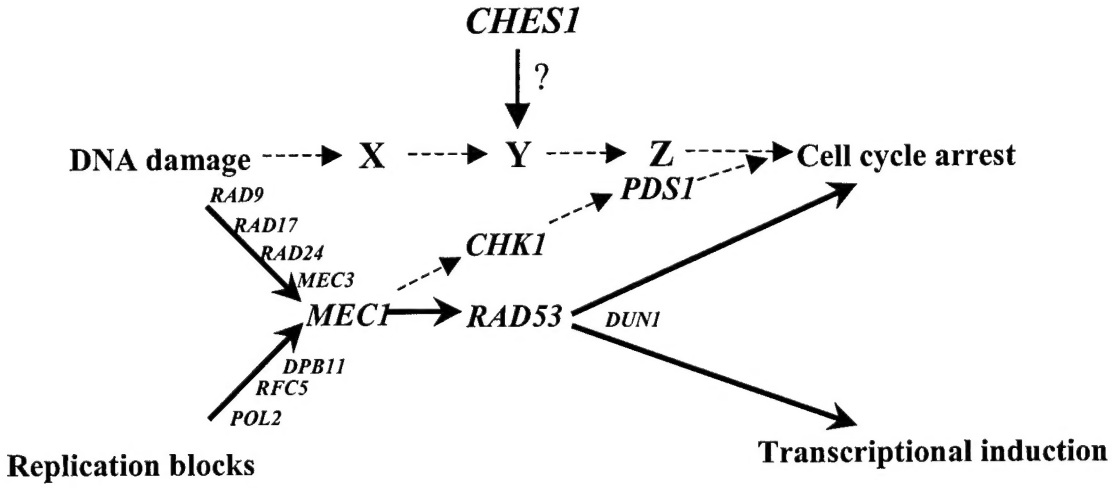

Fig 1. Proposed model for CHES1 action in cell cycle control and response to DNA damage.

Hypotheses

1. An alternative *MEC1*-independent checkpoint pathway, through which *CHES1* reconstitutes G_2 arrest, is present in *S. cerevisiae*. The genes of this pathway can be identified genetically.

2. The alternative checkpoint pathway also exists in other eukaryotes, including mammals, and the function of the constituent genes is to modulate the response of the cell to DNA damage.

Technical Objective 1

Perform a comprehensive mutagenesis screen in *S. cerevisiae* to isolate yeast mutant strains that have defective checkpoint bypass genes

Funding Year 1

The goals for the first year of this grant were to create a temperature-sensitive strain, to optimize mutagenesis conditions, and to perform a mutagenesis screen to identify mutant strains that can no longer be rescued by *CHES1* in the absence of wild type *RAD9*. The method proposed in this project utilizes a temperature-sensitive strain, which contains both the *cdc9-8* and *rad9Δ* mutations. The *rad9Δ* mutation not only lowers the permissive temperature of *cdc9-8* from 30°C to 23°C, but also provides the UV-sensitive phenotype. The *cdc9-8* mutation was gap repaired out from the 9085-1-10-4 strain and put onto an integration vector containing a *URA3* marker. The plasmid was then introduced into a *rad9Δ* strain and plated onto URA deficient- and 5-FOA-containing media sequentially. This allowed isolation of a strain (SCP2) in which the *cdc9-8* allele replaces the wild type allele of *CDC9* in a *rad9Δ* background (*cdc9-8*, *rad9Δ*).

The amount of ethane methyl sulphonate (EMS) required to yield 50% killing in the SCP2 strain was determined to insure optimal mutagenesis conditions. The mutagenesis screen was carried out by a slightly modified method than described in the original grant proposal. Specifically, SCP2 was transformed with two plasmids, each carrying *CHES1* or *RAD9*, before EMS mutagenesis. The transformed strain was mutagenized, followed by washing and plating onto selective media at 30°C. The mutants were replica-plated onto 5-FOA-containing media to select against the *RAD9* plasmid and screened for growth at 23°C but not at 30°C. The colonies identified on this first pass were then patched and replica-plated to retest for temperature-sensitivity. Our model predicts that *chb* (checkpoint bypass) mutants should be not only temperature-sensitive, but also UV-sensitive even when *CHES1* is present in this *cdc9-8*, *rad9Δ* strain. Therefore, positive clones were subjected to a secondary screen to identify those clones

that lost their *CHES1*-dependent response to UV irradiation. The UV sensitivity screen was performed by quantifying the survival rate after exposure to 10 J/m^2 of UV radiation, followed by comparing the isolated *chb* mutants to the parental SCP2 strain transformed with either empty vector or *CHES1*-containing plasmid.

The screen resulted in 310 putative temperature-sensitive mutants of the approximate 220,000 mutagenized clones, and only three clones exhibited true temperature-sensitive phenotypes after retesting. Among those, *chb13* and *chb16* are highly temperature-sensitive whereas *chb57* is a weaker allele. With regard to UV-sensitivity, *chb13* and *chb57* are also sensitive to UV radiation, but the effect of *chb16* is intermediate when compared to the controls (figure 2). The growth of *chb16* is slower than the other two *chb* strains; therefore, *chb16* is a relatively unhealthy mutant in general. Overall, *chb13* appears to be a very strong mutant that has lost all response to *CHES1* by our assays.

The analysis of these mutants has also allowed us to confirm that the *CHES1*-dependent pathway is partially parallel to the *RAD9*-dependent pathway. Our previous data showed that *CHES1* could either act downstream of *DUN1* in the primary *MEC1*-dependent pathway or, more likely, activate an alternative pathway (Pati *et al.*, 1997). Consistent with the parallel pathway model, introduction of a wild type *RAD9* gene restores UV resistance in the SCP2 strain and all three *chb* mutants.

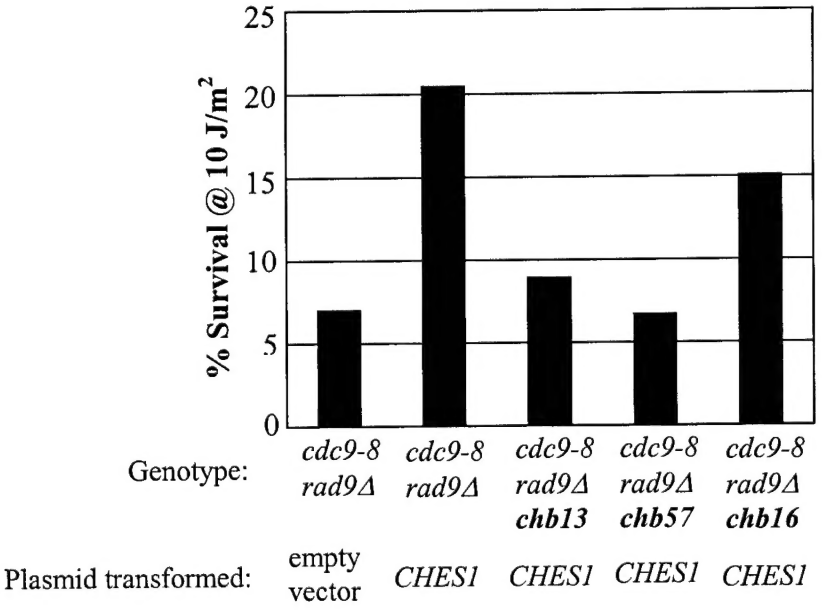

Fig 2. Results of secondary assay of three *chb* mutants identified in the large-scale mutagenesis screen. The percent survival after exposure to 10 J/m^2 is shown for the parental *rad9Δ* strain with a control vector or *CHES1* and the three *chb* mutant strains with *CHES1*.

Funding Year 2

During the second year, we attempted to clone the genes mutated in the characterized *chb* mutants isolated from the EMS mutagenesis screen. We confirmed that all three mutants are recessive by mating to wild type strains. In addition, we determined that all three *chb* mutants belong to the same complementation group (i.e., they all contain mutations in the same gene). Therefore, we decided to clone the gene mutated in *chb13,* which exhibits the strongest phenotype. The *chb13* mutant strain was backcrossed twice and selected for mutant phenotype in the presence of *CHES1* to segregate out any unrelated mutations in the genome. The resulting strain was used for complementation analysis by use of a CEN/TRP yeast genomic library. Thirty-four of the 57,500 transformants screened exhibited high-temperature growth; however, most of these clones contained the *CDC9* genomic fragment, which would be expected from this type of analysis. The remaining clones could not complement the mutant phenotype upon transformation back into the *chb13* mutant strain. Similar results were obtained after screening 20,000 and 36,300 transformants of the *chb57* and *chb16* mutant strains, respectively.

We also took the candidate gene approach by examining two genes that may potentially function in the alternative checkpoint pathway. The *S. pombe* and mammalian homologs of the *S. cerevisiae* Chk1 protein function in response to DNA damage and are necessary for checkpoint function. Therefore, we disrupted *CHK1* in a strain of the same genetic background as in the *chb* mutants. Disruption of *CHK1* did not result in the *chb* phenotype, and over-expression of wild type *CHK1* and *CHES1* in *chb* mutants did not complement the mutant phenotype. The second candidate gene, *TEL1*, is involved in regulating telomere maintenance and is homologous to the *S. cerevisiae* and mammalian checkpoint genes *MEC1* and *ATM*, respectively. Southern blot analysis showed that expression of *CHES1* has no affect on telomere length in either wild type or *tel1Δ* strains. Furthermore, over-expression of wild type *TEL1* and *CHES1* in *chb* mutant strains did not complement the mutant phenotype. Therefore, these data suggest that neither *CHK1* nor *TEL1* are part of the alternative DNA checkpoint pathway.

Problems encountered while studying *CHES1* in the yeast system moved us to begin characterizing *CHES1* in mammalian cells. Northern blot analysis using a set of commercially available tissue-specific RNA blots showed that *CHES1* expression level is low in pancreas, kidney, liver, lung, placenta, brain, heart, testis, prostate, and thymus; medium in colon (mucosal lining), small intestine, and spleen; and high in skeletal muscle, peripheral blood leukocyte, and ovary. Probing a second set of RNA blots demonstrated that *CHES1* is ubiquitously expressed, showing highest expression in the cerebellum, aorta, skeletal muscle, ovary, and adrenal gland. To further examine *CHES1* expression and cellular distribution, antisera against two *CHES1* peptides were generated. However, antibody titers proved to be too low to detect endogenous CHES1 protein.

Funding Year 3

As described above, we were unable to identify the gene(s) mutated in the *chb* mutant strains by complementation and other analyses of these mutants. The segregation pattern for these mutants is complex and may represent more than one mutation in each strain, which may explain the difficulty in cloning the mutations. Based on these results, we made the decision to modify the mutagenesis protocol by performing insertional mutagenesis with the "Snyder" libraries. The strategy involves transforming the SCP2, *CHES1* strain with a library of *S. cerevisiae* genomic clones each containing a *LEU2* marked Tn3 transposon insertion. Individual isolates, which represent homologous recombination events into the genomic locus for each insertion, are selected on media lacking leucine. These transformants will be screened with the same protocol developed for the EMS mutagenesis strategy, and mutant strains resulting from this transformation can be further analyzed for checkpoint defects. The disrupted gene can be directly cloned by use of the *LEU2* marker inserted at the site of the mutation; therefore, we will avoid the problems experienced by the EMS methodology. The insertional mutagenesis protocol requires *CHES1* to be expressed from a plasmid containing a marker other than *LEU2*; therefore, we subcloned *CHES1* into a *TRP1*-containing expression vector (p424). As expected, p424-*CHES1* expressed in SCP2 is sufficient to suppress the temperature-sensitive and UV-sensitive phenotypes exhibited by this strain (figure 3 and data not shown). We have obtained the insertional mutagenesis library and pilot mutagenesis screens are underway.

In addition to modifying the screen mutagenesis protocol, we have moved to identifying proteins that directly interact with the CHES1 protein. In the second year of this grant, a cytoplasmic yeast two-hybrid screen was performed using *CHES1* as the bait and a yeast cDNA library as the prey. Six and a half million yeast transformants were screened and more than 90 positive clones were obtained. After eliminating the bait plasmid from the cells, all of the candidate clones turned out to be bait-independent false positive clones. We recloned the bait construct and are able to observe the ~200 kD fusion protein by Western blot analysis (figure 4); therefore, we will repeat the two-hybrid screen in effort to obtain CHES1-interacting proteins using the functional bait construct.

In addition to the two-hybrid construct, we have constructed a yeast expression vector encoding a GST-CHES1 fusion protein as a means of identifying CHES1-interacting proteins through biochemical methodology. Western blot analysis using anti-GST antibody demonstrates that there is expression of the expected 55kD GST-CHES1 fusion protein (figure 4). Furthermore, the fusion protein retains the ability to suppress the checkpoint defects observed in the SCP2 strain allowing growth at 32°C and increased survival after UV irradiation (figure 3 and data not shown). Protein extracts from yeast expressing either the GST-CHES1 fusion protein or GST alone are being used for GST pulldown assays using Sepharose-glutathione beads. We have successfully enriched for the fusion protein and have produced amounts observable by Coomassie staining. In addition, we are performing the assay using extracts from yeast grown at normal conditions and under conditions that induce DNA damage (32°C) to identify

proteins that interact with CHES1 during the normal cell cycle and after DNA damage, respectively.

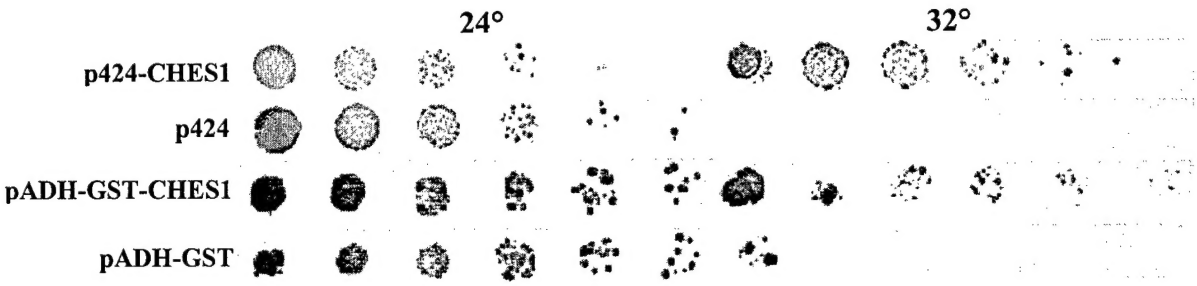

Fig 3. Complementation of a *rad9Δ, cdc9-8* strain by plasmids expressing CHES1 (p424-CHES1) or the GST-CHES1 fusion protein (pADH-GST-CHES1).

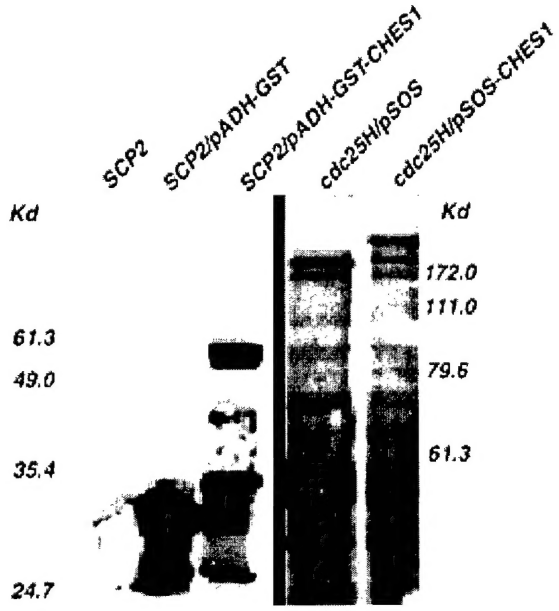

Fig 4. Western blot analysis of yeast lysates transformed with the indicated plasmids and probed with anti-GST or anti-hSOS antibodies.

As discussed above, our effort to create a polyclonal antibody against CHES1 was unsuccessful, as the antibody titers were too low to detect endogenous CHES1 protein. Therefore, we subcloned the C-terminal portion of *CHES1* into a HIS-tagged bacterial expression vector. We are currently optimizing purification and HIS-tag cleavage conditions in an effort to obtain protein for creation of a functional anti-CHES1 antibody.

Technical Objective 2

Searching DNA databases for mammalian homologs of the cloned CHB genes and analysis of CHB gene structure and expression in normal and breast cancer cells.

In the second objective, we proposed to isolate mammalian homologs of the alternative checkpoint pathway and to analyze the structure and expression of the cloned checkpoint genes in human breast cancer derived cell lines and human breast tumor samples. Unfortunately, we were unable to accomplish these goals, as the experiments proposed in the second objective are dependent on data resulting from the first objective. We are optimistic that the changes and new approaches undertaken will result in interesting data in the near future.

Key research accomplishments and reportable outcomes

- Creation of a *cdc9-8*, *rad9Δ* mutant strain (SCP2), which is both UV-resistant and grows at 30°C in the presence of *CHES1*.
- Isolation of three *chb* mutants that display varying degrees of temperature-sensitivity and UV-sensitivity.
- Exclusion of *S. cerevisiae CHK1* and *TEL1* as parts of the alternative DNA checkpoint pathway.
- Elucidation of the CHES1 expression profile by Northern blot analysis.
- Implementation of an alternative mutagenesis strategy, specifically by using insertional libraries to aid in identifying those genes mutated in *chb* mutant strains.
- Construction of a functional hSOS-*CHES1* fusion vector for use in a cytoplasmic yeast-two-hybrid screen currently in progress.
- Construction of a functional GST-*CHES1* fusion vector for use in GST pulldown assays currently in progress.
- Construction of a HIS-tagged *CHES1* bacterial expression vector for creation of a functional anti-CHES1 polyclonal antibody.
- Presentations:

  S. E. Plon, D. Pati, and Y.-C. Li. Alternative DNA damage checkpoint pathways in eukaryotes. Poster presentation. Ataxia Telangiectasia and ATM: Functional, Genetic and Clinical Ramifications, Baltimore, Maryland, August 1997.

  Y.-C. Li and S. E. Plon. Isolation of mutants that are defective in an alternative DNA damage checkpoint pathway. Poster presentation. American Society for Microbiology Conference: Yeast Genetics and Human Disease II. Vancouver, British Columbia, Canada. June 24 – 27, 1999.

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